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Drug discovery for X-linked adrenoleukodystrophy: An unbiased screen for compounds that lower very long-chain fatty acids

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Abstract

X-linked adrenoleukodystrophy (XALD) is a genetic neurologic disorder with multiple phenotypic presentations and limited therapeutic options. The childhood cerebral phenotype (CCALD), a fatal demyelinating disorder affecting about 35% of patients, and the adult-onset adrenomyeloneuropathy (AMN), a peripheral neuropathy affecting 40%–45% of patients, are both caused by mutations in the *ABCD1* gene. Both phenotypes are characterized biochemically by elevated tissue and plasma levels of saturated very long-chain fatty acids (VLCFA), and an increase in plasma cerotic acid (C26:0), along with the clinical presentation, is diagnostic. Administration of oils containing monounsaturated fatty acids, for example, Lorenzo's oil, lowers patient VLCFA levels and reduced the frequency of development of CCALD in pre-symptomatic boys. However, this therapy is not currently available. Hematopoietic stem cell transplant and gene therapy remain viable therapies for boys with early progressive cerebral disease. We asked whether any existing approved drugs can lower VLCFA and thus open new therapeutic possibilities for XALD. Using SV40-transformed and telomerase-immortalized skin fibroblasts from an XALD patient, we conducted an unbiased screen of a library of approved drugs and natural products for their ability to decrease VLCFA, using measurement of C26:0 in

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AUTHOR CONTRIBUTIONS

Steven J. Steinberg and **Paul A. Watkins:** conceived and designed the study. **Nancy E. Braverman:** transformed and immortalized the fibroblast cell lines used. **Yanqiu Liu, Xiaohai Shi, Ulrike Schrifl**, and **Shandi Hiebler:** conducted the experiments. **Ann B. Moser:** performed all LC-MS/MS analyses. **Paul A. Watkins, Ann B. Moser**, and **Ali Fatemi:** wrote the manuscript. **Paul A. Watkins** and **Steven J. Steinberg:** obtained funding for the study.

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CONFLICT OF INTERESTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

lysophosphatidyl choline (C26-LPC) by tandem mass spectrometry as the readout. While several candidate drugs were initially identified, further testing in primary fibroblast cell lines from multiple CCALD and AMN patients narrowed the list to one drug, the antihypertensive drug irbesartan. In addition to lowering C26-LPC, levels of C26:0 and C28:0 in total fibroblast lipids were reduced. The effect of irbesartan was dose dependent between 2 and 10 μM. When male XALD mice received orally administered irbesartan at a dose of 10 mg/kg/day, there was no reduction in plasma C26-LPC. However, irbesartan failed to lower mouse fibroblast C26-LPC consistently. The results of these studies indicate a potential therapeutic benefit of irbesartan in XALD that should be validated by further study.

Keywords

drug discovery; fibroblasts; irbesartan; very long-chain fatty acids; X-linked adrenoleukodystrophy

1 | INTRODUCTION

X-linked adrenoleukodystrophy (XALD) is the most commonly encountered peroxisomal disorder, with an allele frequency of at least 1:17,000.[1] While hemizygous males are predominantly affected, female carriers often show age-related symptoms.[2] Mutations in the ABCD1 gene, which encodes the adrenoleukodystrophy protein (ALDP), have been found in all XALD patients.[3]

The clinical presentation of XALD is variable.[4] Childhood cerebral XALD (CCALD) is the most severe phenotype, and accounts for about 35% of affected boys. Boys with CCALD are typically asymptomatic until about 5–7 years of age, at which time they show behaviors reminiscent of attention deficit disorder. This is followed by symptoms of leukodystrophy, including progressive behavioral changes, loss of vision and hearing, motor dysfunction, and dementia. Nearly all boys with CCALD have adrenocortical insufficiency (Addison's disease). If untreated, CCALD is usually fatal within about 2 years after onset of neurological symptoms. While a small percentage (~10%) of XALD males have Addison's disease only, most patients (40%–45%) are adult men with adrenomyeloneuropathy (AMN). Unlike CCALD, AMN is a slowly progressive myelopathy and peripheral neuropathy. Onset of AMN in males is usually in early adulthood, and typically presents as a gait disorder with sensory ataxia and progressive spasticity and weakness of the lower extremities, as well as bowel and bladder control issues, and sexual dysfunction. Many men with AMN live for decades without cerebral manifestations of the disease; however, at least 20% go on to develop adult cerebral XALD (ACALD) which can often have a rapid progressive course similar to CCALD. Importantly, while adrenal insufficiency and cerebral are extremely rare in female *ABCD1* mutation carriers, at least half of all female heterozygotes will also develop AMN, but the age of onset is typically later than in males, and the phenotype is less severe.

From a biochemical perspective, XALD is a disorder of very long-chain fatty acid (VLCFA) metabolism.[5,6] All XALD patients, irrespective of phenotype, have elevated levels of saturated VLCFA in plasma and tissues. Increased plasma cerotic acid (C26:0) in a patient

suspected of having XALD is diagnostic. VLCFA are found in complex lipids throughout the body and are particularly abundant in central nervous system myelin and the adrenal cortex.[7] VLCFA levels are elevated in utero and remain elevated throughout life in untreated patients.

VLCFA levels are regulated in part by degradation of excesses via β-oxidation in peroxisomes.[8,9] To be degraded, VLCFA must first be activated to their coenzyme A (CoA) thioesters. It is currently held that this process occurs outside the peroxisome, and that ALDP, a peroxisomal integral membrane protein belonging to the ATP-binding cassette (ABC) family of transporters, is required to transport VLCFA-CoA across the membrane and into the matrix where β-oxidation pathway enzymes are located.[10] Mutations in ALDP prevent transport, and therefore degradation, of excess VLCFA.

Very limited curative treatment options exist for XALD. The only effective therapy currently is allogeneic hematopoietic stem cell transplantation (HSCT) which is effective in halting progression of CCALD when performed in neurologically asymptomatic boys with early brain magnetic resonance imaging (MRI) lesions.[11,12] However, this procedure is associated with a high rate of morbidity due to complications related to allogeneic transplantation. A more recent approach, which is still under investigation, utilized ex-vivo lentiviral transduction of autologous bone marrow stem cells and results from the initial trial in boys with CCALD show comparable results to standard allogeneic HSCT.[13] No effective therapies exist for AMN or ACALD.

Dietary Lorenzo's oil (LO), a mixture of triglycerides of oleic (C18:1ω9) and erucic (C22:1ω9) mono-unsaturated fatty acids, was shown to lower plasma saturated VLCFA levels in XALD patients.[14] In a single-arm open label preventive trial of LO, treating neurologically asymptomatic XALD boys with no evidence of demyelination on brain MRI, those individuals who normalized their plasma VLCFA levels appeared to have a lower chance of developing CCALD compared to historical data.[15] However, several other trials in symptomatic XALD patients with different phenotypes have failed to show any efficacy of LO and significant adverse side effects were noted.[16,17]

We, therefore, asked whether there are any existing drugs, or drug-like compounds, that could serve a similar, VLCFA-lowering, function. To this end, we performed unbiased screening of a library of Food and Drug Administration-approved drugs and natural products for their ability to lower VLCFA in transformed and immortalized skin fibroblasts from an XALD patient. Although total lipid VLCFA content of fluids, tissues, or cells is typically measured by gas chromatography-mass spectrometry, an alternative assay was developed to be more compatible with newborn screening of dried blood spots. This assay measures C26:0-lysophosphatidylcholine (C26-LPC) by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).[18,19] Measurement of this metabolite has proven to be particularly robust, and we have employed it in this work.

In this report, we show that the antihypertensive drug, irbesartan, consistently and statistically significantly lowers C26-LPC, and total VLCFA levels, in fibroblasts from multiple CCALD and AMN patients. While the drug, when administered orally to XALD

mice, did not lower plasma C26-LPC, it also did not consistently lower this VLCFA in XALD mouse fibroblasts. We believe this to be the first report of an unbiased drug screen to identify drugs that may be of benefit in the therapy of XALD.

2 | MATERIALS AND METHODS

2.1 | Materials and general methods

The Johns Hopkins Clinical Compound Library[20] was the kind gift of Dr. Jun O. Liu (Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine). Drugs selected for further study were from Sigma Aldrich, except for irbesartan, which was from AK Scientific. Cell culture reagents were all from Corning Cellgro, except for fetal bovine serum (FBS), which was obtained from Biosource International. Protein was determined by the method of Lowry et al.[21]

2.2 | Primary fibroblasts and culture

All procedures were approved by the Johns Hopkins Institutional Review Board. Skin fibroblasts from patients were originally obtained either at other institutions and sent to the Kennedy Krieger Institute, or at the Kennedy Krieger Institute or Johns Hopkins University School of Medicine, for diagnostic purposes. Informed consent was obtained to use cells for research as well as diagnostic purposes. Normal control fibroblasts were obtained from the NIGMS cell repository, Coriell Institute, Camden, NJ. Cells were stored in liquid nitrogen as part of our Intellectual and Developmental Disabilities Research Center. Fibroblasts were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in minimal essential medium (MEM) supplemented with 10% FBS.

2.3 | SV40 transformation and culture conditions

One control fibroblast line (Coriell GM09503, derived from a normal male), and one XALD line (AMN-7, derived from a patient with *ABCD1* mutation c.1415_1416del [p.E471fs]) were transformed with SV40 large-T antigen as described[22] and immortalized with telomerase pBabePuro/hTERT.[23] Immortalized fibroblasts were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. The immortalized control and XALD lines are designated GM09503*E* and AMN-7*E*, respectively.

2.4 | Medium-throughput drug screen

Screening was carried out in standard 96-well culture plates. We screened the Johns Hopkins Clinical Compound Library,[20] which consists of 2560 compounds in 96-well format. Normal control cells were plated in wells A-D of columns 1 and 12; XALD cells were plated in all other wells. Immortalized cells were plated at a density that would reach confluence in 4 days. One day after plating, vehicle (dimethyl sulfoxide [DMSO], final concentration 0.1%) was added to all wells of columns 1 and 12 (4 control and 4 XALD wells in each column), and drug (final concentration 10 μM) in DMSO was added to wells in columns 2–11. After 3 days of exposure to drug, plates were processed for quantitation of C26-LPC. For primary skin fibroblasts, a longer treatment protocol was used. Control and XALD cells were plated at a density that would reach confluence at 10 days of culture. One

day after plating, vehicle or drug was added as for immortalized cells. On Days 3, 6, and 8, medium was replaced with fresh medium containing either vehicle or drug. Plates were processed on Day 10.

After incubation of cells with drug, culture medium was aspirated and cells were washed twice with 200 µl Hank's balanced salt solution (Corning Cellgro). Cells were solubilized in 25 µl Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) by shaking for 1 h at 4°C. A total of 150 µl of methanol containing internal standard ($^2H_4$-C26-LPC; Avanti Polar Lipids) was added to each well. Plates were covered with a cap mat and shaken for 1 h at room temperature. Contents of each well were transferred to a 96-well filter plate (Pall) which was placed on a deep-well collection plate (Acros). Plates were centrifuged for 10 min at 2000 rpm. Deep-well plates were covered with cap mats and stored at −20°C pending LC-MS/MS analysis.

2.5 | Assessment of screen quality

Plate columns 1 and 12 contained normal control and XALD fibroblasts that were treated with vehicle alone and were used to determine the upper (XALD) and lower (normal control) C26-LPC limits. These values were used to calculate Z-factor (Z′), defined as: $Z' = 1 - (3 \times SSD/R)$, where R = absolute mean difference and SSD = sum of the SDs.[24] A Z-factor of 0.5–1.0 is excellent, while 0–0.5 is good and less than 0 is unacceptable.

2.6 | Lipid analyses

C26-LPC was quantitated in methanol extracts of cells cultured in 96-well plates by LC-MS/MS as previously described.[25] For total fatty acid analysis of irbesartan-treated primary fibroblasts, cells were cultured in 6 cm diameter culture dishes. Following the 10-day treatment protocol, total fatty acids were quantitated by gas chromatography-mass spectrometry as previously described.[26]

2.7 | Animal studies

All studies were approved by the Johns Hopkins Animal Care and Use Committee. Generation of the XALD mouse model by knockout of the ABCD1 locus was previously reported.[27] XALD mice were maintained on the 129SvE background as hemizygous males and homozygous females. Wild-type mice of the same background strain were purchased from Taconic Biosciences.

For oral administration of irbesartan to mice, the drug was dissolved in ethanol and adsorbed to broken pieces of standard laboratory chow. Before the start of the study, it was determined that mice of this age consumed approximately 3.5 g chow/day. It was also determined that 0.37 ml ethanol per gram chow was sufficient to coat the food thoroughly, but without residual unabsorbed liquid. Based on these values, when adsorbed to chow, 0.21 mg irbesartan/ml ethanol will deliver a nominal dose of 10 mg irbesartan/kg body weight/day. Chow was mixed with drug in ethanol by stirring until pieces were evenly coated. Chow was placed in a glass pan in a fume hood to allow solvent evaporation overnight. Control chow was similarly prepared by mixing chow with ethanol alone. Fresh chow was prepared and supplied every 3–4 days.

Ear clippings of XALD male and female mice, and wild-type mice were used to generate skin fibroblasts as previously described.[27] Mouse fibroblasts were maintained in DMEM with 10% FBS. For assessment of irbesartan's effect on C26-LPC levels, the 96-well, 10-day protocol for human primary fibroblasts was followed.

2.8 | Statistical methods

Results are presented as mean ± *SD*. Statistical significance was determined using unpaired, two-tailed Student's *t* test. The *p* values are designated as follows: $*p < .05$; $**p < .01$; $***p < .001$.

3 | RESULTS

3.1 | Fibroblast C26-LPC level is a valid biomarker for XALD drug screening

Measurement of total lipid C26:0 has been the accepted method for assessing VLCFA content of plasma, tissues, or cells from XALD patients.[6] This gas chromatography-mass spectrometry-based method clearly distinguishes primary normal control fibroblasts from XALD patient fibroblasts (Table 1). However, this method is time- and labor-intensive, and thus not readily adaptable for drug library screening. Therefore, we investigated the possibility of using C26-LPC, measured by LC-MS/MS, as an alternative biomarker. As shown in Table 1, this assay was as robust as total lipid VLCFA for discriminating between control and XALD primary fibroblasts.

3.2 | Transformed XALD fibroblasts are suitable for drug screening in 96-well format

To avoid potential problems introduced by senescence of primary skin fibroblasts in long-term culture, one control line (GM09503) and one XALD line (AMN-7) were transformed with SV40 large-T antigen and immortalized using telomerase as described in Methods. The immortalized control and XALD cell lines were designated GM09503*E* and AMN-7*E*, respectively. We then tested whether C26-LPC content could discriminate between XALD and control immortalized fibroblasts grown on 96-well plates. C26-LPC concentrations were 10.9 ± 2.5 and 43.1 ± 5.7 pmol/mg protein in GM09503*E* and AMN-7*E* cells, respectively (Table 1). These values were similar to the results with non-immortalized cells, in which the C26-LPC concentrations were 6.72 ± 1.92 and 45.3 ± 16.8 pmol/mg protein in GM09503 and AMN-7 cells, respectively. In the immortalized XALD fibroblasts, there was a fourfold higher C26-LPC concentration than in the immortalized control fibroblasts.

3.3 | First-round screening of immortalized fibroblasts identified 42 candidate drugs

For rapid translation to clinical use in humans, repurposing of approved drugs is an attractive approach. We chose to screen the Johns Hopkins Clinical Compound Library,[20] which consists of 2560 compounds in 96-well format. Most are approved drugs (United States, Europe, Asia, Australia) although some are natural products or are drugs in clinical trials.

The overall screening strategy is outlined in Figure 1. Because the screen is at best medium-throughput, to identify candidate drugs efficiently, we chose to assay the library in singlicate, and without normalization for cellular protein, while recognizing the inherent limitations of these choices. Immortalized XALD fibroblasts were plated in 96-well plates.

After attachment, they were treated with drugs/compounds at a final concentration of 10 μM for 3 days, after which C26-LPC was extracted and quantitated. Before extraction, all plates were evaluated microscopically. Wells containing cells that were morphologically altered (e.g., rounded), floating, or significantly reduced in number were excluded from consideration. Thirty two 96-well drug library plates were assayed in the initial round of screening. A total of 59% had excellent (0.5–1.0) Z-factors, and 31% were in the good (0–0.5) range. The 10% that were in the unacceptable (<0.0) range were discarded, and the procedure was repeated; Z-factors for all repeat analyses were good to excellent. Drugs that reduced the C26-LPC levels of XALD cells more than 50% of the way toward control C26-LPC levels were considered to be candidates for further evaluation. Representative first-round screening data from a single plate is shown in Figure 2. For this plate, only one drug (circled) lowered C26-LPC more than 50%. Out of 2560 compounds on 32 plates, 42 compounds satisfied this criterion. Interestingly, several drugs increased the C26-LPC level (Figure 2).

3.4 | Further screening of immortalized fibroblasts identified four candidate drugs

Immortalized XALD fibroblasts were subjected to additional screening with the compounds identified in the first round using single or multiple wells of 96-well plates. Again, drug treatment was at a final concentration of 10 μM for 3 days. Only 10 of the 42 drugs significantly lowered C26-LPC in a single-pass repeat screening. Because our goal was to identify existing drugs that could be repurposed for treating boys with CCALD or men with AMN, we eliminated four drugs that were unsuitable for long-term therapy. These included a contrast agent (iopanic acid), a topical antimicrobial (clobetasol), a preservative (benzalkonium chloride) and a drug not approved for use in children (milnacipran). The six remaining drugs—lobetalol (α,β-blocker), dicoumarol (anticoagulant), naltrexone (opioid antagonist), papaverine (anti-spasmodic), risedronate (osteoporosis), and thalidomide (leprosy, myeloma)—were tested on immortalized XALD fibroblasts in octuplicate wells of a 96-well plate. Only three drugs, dicoumarol, papaverine, and risedronate, caused a statistically significant reduction in VLCFA levels (Figure 3).

Careful inspection of the screening data indicated that two additional drugs, betamethasone and irbesartan, deserved scrutiny. Both drugs were present on more than one 96-well plate, but under different names. Betamethasone was on plate 3 (as both betamethasone valerate and betamethasone sodium phosphate) and on plate 4 (as betamethasone). Irbesartan was present on plate 11 (as generic irbesartan) as well as on plate 22 under a trade name. While neither drug lowered C26-LPC on the same plate more than 50% in both the first and repeat assays, both drugs did so on at least one pass for all wells containing them. Betamethasone is a topical corticosteroid also available as an injectable; however, the latter formulation can be immunosuppressive. Thus, we eliminated betamethasone and included irbesartan in subsequent studies with primary XALD fibroblasts.

3.5 | Assessment of C26-LPC-lowering drugs in cultured primary skin fibroblasts

We next determined whether the candidate drugs would lower VLCFA levels in primary fibroblasts as well as immortalized fibroblasts. Because the growth rate of primary fibroblasts is significantly slower than that of immortalized cells, we increased the time

of drug exposure to 10 days (see Section 2). We then tested four of the drugs from Figure 3—three that significantly lowered VLCFA and one that did not—in primary fibroblasts from two CCER XALD patients and one AMN patient. In agreement with data from immortalized fibroblasts, dicoumarol and papaverine lowered C26-LPC levels in primary fibroblasts by an average of 33% and 41%, respectively (Table S1). In contrast to immortalized cell findings, risedronate was ineffective, while thalidomide showed a modest reduction in primary fibroblast C26-LPC. Based on these results, risedronate was eliminated from consideration. Further testing was done using dicoumarol, papaverine, thalidomide, and irbesartan.

To correct for any changes in cell growth during the 10-day drug treatment, duplicate 96-well plates were prepared and treated with drug. One plate was analyzed for C26-LPC, and the other was used for cell protein determination. Dicoumarol, papaverine, thalidomide, and irbesartan were tested in primary fibroblasts from several XALD patients. When corrected for effects on cellular protein, promising candidate drugs dicoumarol, papaverine, and thalidomide did not consistently lower C26-LPC levels (Table 2), while irbesartan consistently did so (Table 2 and Figure 4). A total of 10 μM irbesartan lowered the C26-LPC level by an average of 40% (range: 22%–67%) in fibroblasts from six CCER, one adolescent cerebral, and six AMN patients. With the exception of the adolescent cerebral XALD patient, VLCFA reductions were all statistically significant (Table 2 and Figure 4). When tested in primary fibroblasts from four XALD patients, the effect of irbesartan on C26-LPC was dose-dependent in the range of 2–10 μM (Figure 5).

3.6 | Irbesartan lowers total lipid VLCFA levels in primary XALD fibroblasts

To verify that irbesartan effects were not unique to C26-LPC, but were reflective of total VLCFAs, primary fibroblasts from five AMN patients and one normal control were incubated without or with the drug for 10 days. Total lipid VLCFA was measured by GC-MS. As shown in Figure 6, the total C24:0 level in untreated XALD cells was not elevated with respect to control fibroblast levels. In contrast, C26:0 and C28:0 levels were more than fivefold higher in untreated XALD fibroblasts. Irbesartan consistently lowered both C26:0 and C28:0 levels in patient cells. Interestingly, the drug had either no effect, or caused a minor elevation of the noninformative VLCFA, C24:0. These results indicate that measurement of C26-LPC is a sensitive surrogate for monitoring drug effects on the VLCFAs that are elevated in XALD fibroblasts.

3.7 | Irbesartan did not lower plasma C26-LPC in XALD mice

Encouraged by the consistent VLCFA-lowering effect of irbesartan in patient fibroblasts, we conducted an oral feeding study in XALD mice. Four wild-type male and four XALD hemizygous male mice were fed chow to which irbesartan had been adsorbed, calculated to deliver a dose of 10 mg/kg/day, for 6 weeks. Over the course of the study, body weight remained constant in both groups, indicating that the drug was consumed. As shown in Figure 7, there was no effect of irbesartan on plasma C26-LPC levels at any time point.

3.8 | Irbesartan did not consistently lower C26-LPC in XALD mouse fibroblasts

To determine whether lack of irbesartan effects in mice in vivo were potentially due to species differences, we assessed the effect of drug on primary mouse skin fibroblasts. Cells

from wild-type male, homozygous XALD female, and hemizygous XALD male animals were treated with irbesartan using the same 10-day protocol used with primary human fibroblasts. As shown in Figure 8, effects of drug on C26-LPC levels were inconsistent. As expected, C26-LPC levels in untreated fibroblasts were consistently higher in male XALD versus wild-type mouse fibroblasts; levels in female XALD cells were intermediate. In two of three studies, irbesartan had no significant effect on C26-LPC levels in male XALD mouse fibroblasts; however, on one occasion there was a significant ($p < .01$) decrease. In two of three studies with female XALD cells, the effect of the drug was a statistically significant ($p < .001$) increase, rather than decrease, in C26-LPC levels. Taken in total, we conclude that the consistent and statistically significant decrease in fibroblast C26-LPC levels observed with human cells is not reproduced in mice, at least at the tested dose of 10 mg/kg/day. These observations suggest that the lack of effects of orally administered irbesartan in mice may be due either to species differences, or to a suboptimal dose.

4 | DISCUSSION

Elevated plasma and tissue levels of VLCFA are considered to be the biochemical hallmark of XALD.[5,6] Several studies have explored the impact of impaired VLCFA metabolism on various cellular pathways. VLCFA stimulation of CCALD patient macrophages results in a dramatic proinflammatory response of these cells along with reduction of antioxidant capacity.[28] In addition, disruption of oxidative phosphorylation has been shown to correlate with VLCFA dose increase in ABCD1 deficiency fibroblasts.[29,30] Impaired autophagy,[31] increase in endoplasmic reticulum stress[32] and impaired endothelial tight junction functions[33] have also been observed as a consequence of VLCFA increase in ABCD1 mutant cells. Therefore, lowering of the body burden of VLCFA will likely have a favorable effect on disease progression and thus quality of life. Thus various strategies targeting either VLCFA synthesis or μ-oxidation are currently being explored in XALD. In this study, we conducted an unbiased screen of approved drugs, and found that the anti-hypertensive drug irbesartan consistently lowered VLCFA in XALD fibroblasts.

There are two sources of VLCFA in humans—dietary intake, or elongation of long-chain (16-to 18-carbon) fatty acids in the endoplasmic reticulum. VLCFA homeostasis is achieved by degradation of excesses via peroxisomal β-oxidation. Unlike mitochondrial fatty acid β-oxidation, in which fatty acids are catabolized for energy production, peroxisomal β-oxidation serves in a detoxification (e.g., regulation of VLCFA levels) or synthetic (e.g., docosahexaenoic acid biosynthesis) capacity. ALDP is a peroxisomal integral membrane protein belonging to the ABC family of transporters. It is now thought that VLCFA are activated to their CoA thioesters outside the peroxisome, and that ALDP is required to transport VLCFA-CoA across the membrane and into the matrix where the β-oxidation pathway enzymes are located. When ALDP is mutated, as in XALD, the excess VLCFA-CoA become substrates for synthesis of complex lipids such as phospholipids, sphingolipids, and cholesterol esters. Investigation of the mechanism by which irbesartan lowers VLCFA is beyond the scope of the present investigation. Although it is likely that the drug either decreases fatty acid elongation or stimulates VLCFA β-oxidation, further experiments are needed to rule in or out these or other mechanisms.

Several drugs have been investigated for their ability to lower saturated VLCFA levels in XALD. LO significantly lowered plasma VLCFA levels, but while one single-arm open label trial appeared to show a protective effect,[15] several other trials failed to show any efficacy. However, an autopsy study of XALD patients treated with LO, showed that only one out of four subjects had reduction of VLCFA in the brain. It is therefore necessary to identify compounds that can penetrate the blood brain barrier and can lower VLCFAs in the CNS.

Based on the early observation that a second member of the ABCD gene family, ABCD2, can partially functionally compensate for the lack of ABCD1 in XALD cells and tissues,[34,35] several investigators looked at drugs that upregulate ABCD2 expression. These include the AMP kinase activator, metformin,[36] caffeic acid phenethyl ester,[37] several LXR antagonists,[38] thyroid hormone,[39] and a thyromimetic, sobetirome.[40] All of these drugs except the LXR antagonists lowered VLCFA in human XALD fibroblasts, or XALD mice. Of this group, thus far only sobetirome has been administered to humans (clinicaltrials.gov), but results are not yet published. In addition to upregulating ABCD2, caffeic acid phenethyl ester upregulated peroxisomal VLCFA β-oxidation and reduced expression of ELOVL1, the rate-limiting step in fatty acid elongation.[37]

Bezafibrate, which does not upregulate ABCD2, lowers XALD fibroblast VLCFA by inhibiting ELOVL1.[41] However, a clinical trial involving 10 AMN patients receiving up to 800 mg/day failed to show lowering of VLCFA in plasma or lymphocytes.[42] The histone deacetylase inhibitor suberoylanilide hydroxamic acid, also lowered VLCFA levels in XALD fibroblasts by upregulating peroxisomal VLCFA β-oxidation and reducing expression of ELOVL1, but independently of ABCD2.[43–45] The PPARγ agonist pioglitazone has also been investigated for its therapeutic potential in XALD; this drug lowers markers of oxidative stress observed in XALD without directly lowering VLCFA levels.[46] A clinical trial of pioglitazone was reported as completed, but results are not available yet (clinicaltrials.gov). A clinical trial of a related PPARγ agonist, leriglitazone (MIN-102) is listed as active by clinicaltrials.gov.

Irbesartan is one of many “sartan” drugs that block angiotensin II receptors.[47] Interestingly, irbesartan was the only member of this family that lowered VLCFA in our drug screen. Because XALD is a neurodegenerative disease, the ability to cross the blood-brain barrier is a desirable property. Michel et al.[47] reviewed the properties of angiotensin II type 1 receptor blockers, including their ability to enter the brain, and the results of several studies suggest that orally administered irbesartan has a modest ability to affect CNS physiology. The blood-brain barrier is disrupted in CCALD patients,[33] potentially rendering these individuals more amenable to irbesartan entry into the CNS than AMN or presymptomatic patients.

The mechanism by which irbesartan lowers VLCFA in XALD fibroblasts remains under investigation. Upregulation of ABCD2, stimulation of VLCFA β-oxidation, and inhibition of ELOVL1 are possibilities, as other VLCFA-lowering drugs mentioned above likely affect these pathways. Other mechanisms, such as increased activation of VLCFA substrates to their CoA derivatives, must also be considered. Although several C26-LPC-lowering drugs

were ruled out as inappropriate for longterm therapy of XALD, further investigation of their mechanism(s) of action may help identify new potential molecular targets for XALD.

Oral irbesartan treatment did not lower plasma C26-LPC levels in the XALD mouse. Our observation that this drug did not consistently lower VLCFA levels in male XALD mouse primary fibroblasts, and increased levels in homozygous female mouse cells, suggests there may be species differences in response to the drug. It is also possible that the dose (10 mg/kg/day), route of administration, or duration of treatment were inappropriate. In particular, the chosen dose of irbesartan may be too low. Other investigators have used up to 50 mg/kg/day to achieve biological effects,[48,49] Additional studies using a higher dosage of irbesartan in the XALD mouse model are thus needed. We conclude that the lack of response in mice should not rule out exploration of the use of irbesartan in humans.

Irbesartan is widely used as an anti-hypertensive drug with very limited side effect profile and is generally well tolerated. Pharmacokinetic studies have been performed extensively and proof of concept trial in adult men with AMN would be relatively easy to conduct to determine if treatment for several weeks to months can lower plasma VLCFA levels. While, further work is needed to understand the mechanism of action of irbesartan on VLCFA metabolism in XALD, a pilot human trial may be a feasible path forward for XALD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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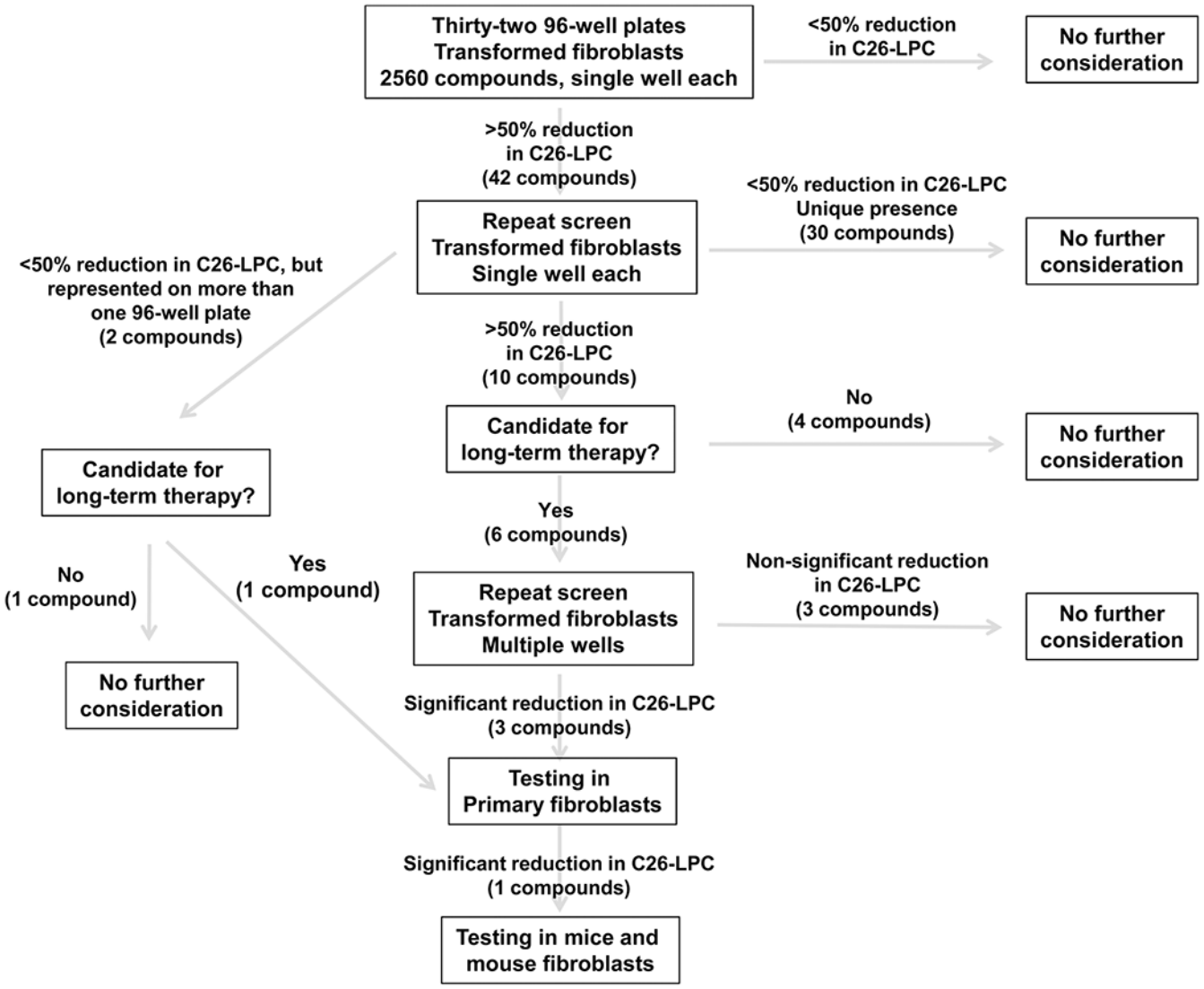

FIGURE 1.

Screening strategy for drugs that lower C26-LPC in immortalized XALD fibroblasts. This flow chart depicts the screening strategy, and outcome at each step is shown. In the initial screen of 32 plates, and the repeat screen, microscopy was used to eliminate drugs that adversely affected morphology, attachment, or cell growth.

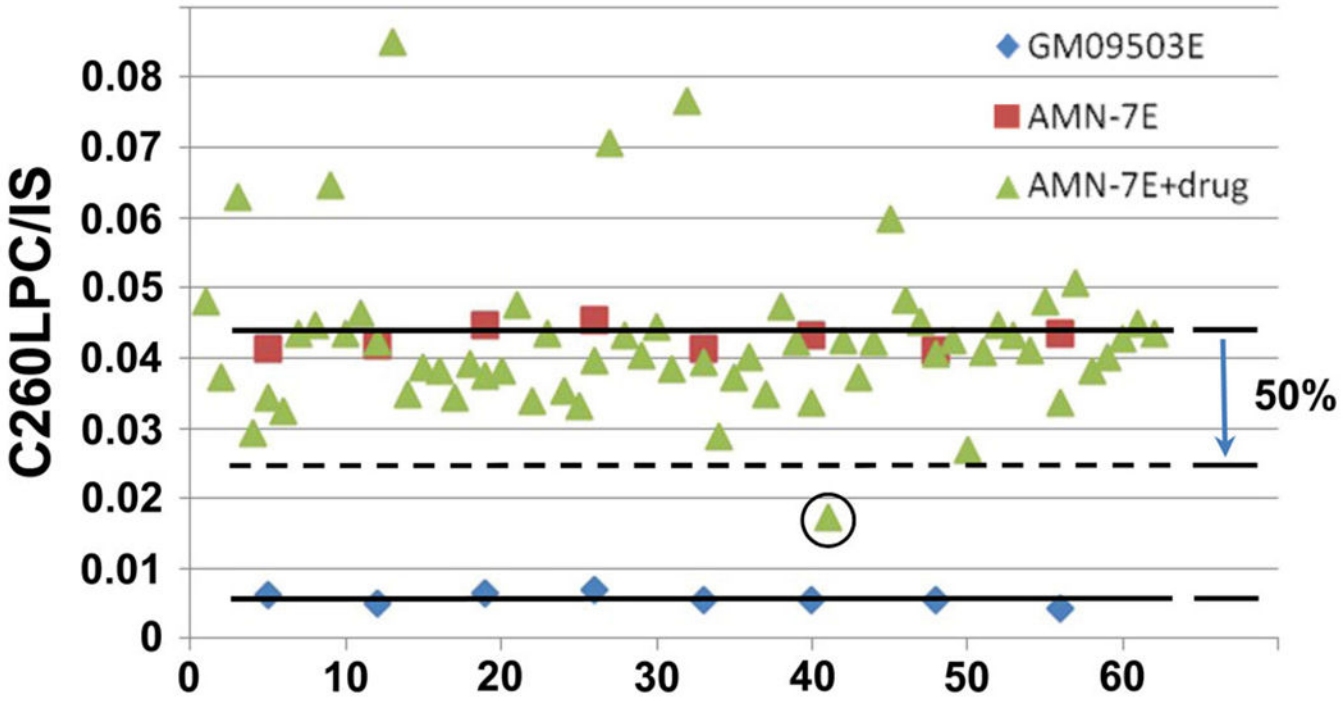

FIGURE 2.

Screening criteria. For all 96-well drug screening analyses, 8 wells contained vehicle-treated immortalized control (GM09503*E*) cells, and 8 wells contained vehicle-treated immortalized XALD (AMN-7*E*) fibroblasts. The remaining 80 wells contained drug-treated AMN-7*E* cells. C26-LPC levels were measured and normalized to an internal standard (IS). In this representative study, 18 of the 80 compounds present on the 96-well plate were ruled out by microscopic assessment. C26-LPC levels for 62 drug-treated wells are shown as green triangles. Mean values for control (blue diamonds) and XALD cells (red squares) are depicted by solid lines. The dashed line represents the value for a 50% reduction in C26-LPC toward normal. Only 1 compound (circled) lowered C26-LPC more than 50%. The Z-factor for this analysis was 0.85. XALD, X-linked adrenoleukodystrophy

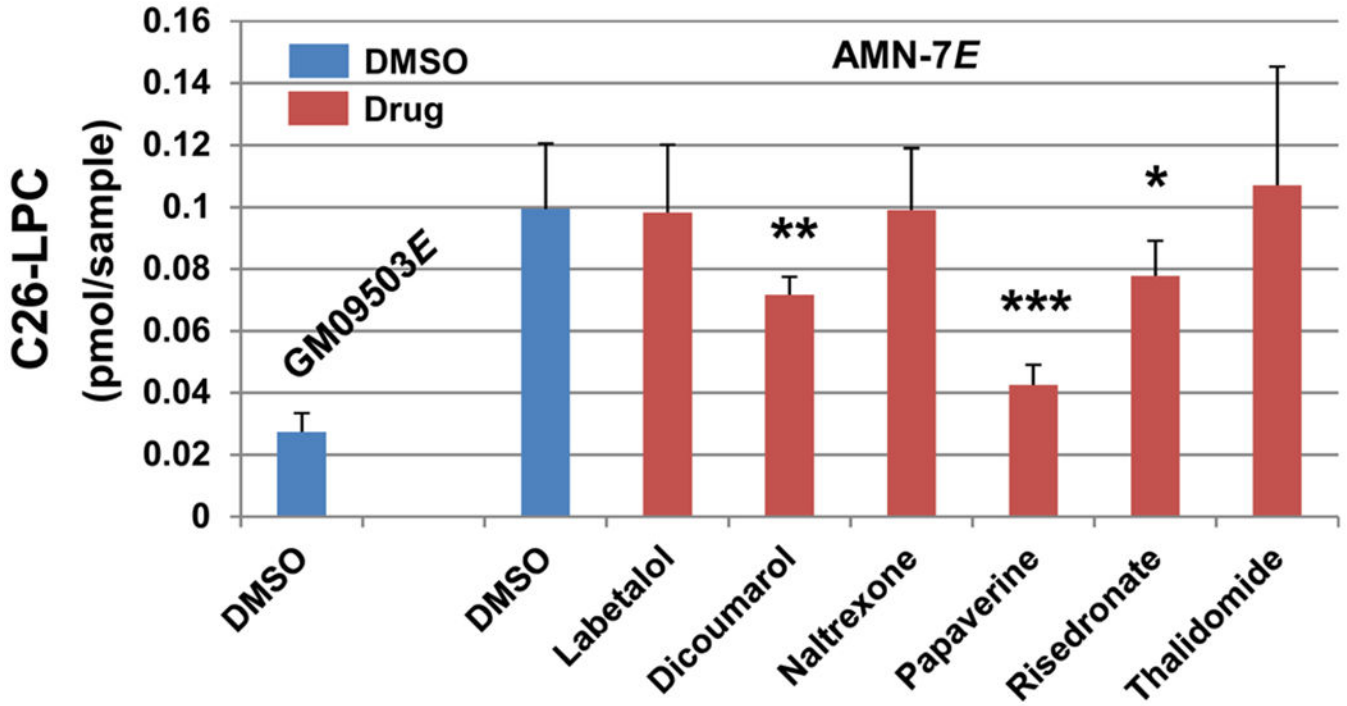

FIGURE 3.

Effect of candidate drugs on C26-LPC levels in immortalized XALD fibroblasts (AMN-7*E*). Several drugs identified in two rounds of screening were further assessed in immortalized XALD fibroblasts. Quadruplicate wells of a 96-well plate were treated with 10 μM drug or vehicle (DMSO) for 3 days, followed by quantitation of C26-LPC, as described in Methods. Immortalized normal control GM09503*E* cells were treated with DMSO alone. C26-LPC concentration (mean ± *SD*) is shown; values were not normalized to cell protein. Relative to DMSO alone, dicoumarol, papaverine, and risedronate caused a statistically significant lowering of C26-LPC in the AMN-7*E* cell line (*$p < .05$; **$p < .01$; ***$p < .001$). DMSO, dimethyl sulfoxide; XALD, X-linked adrenoleukodystrophy

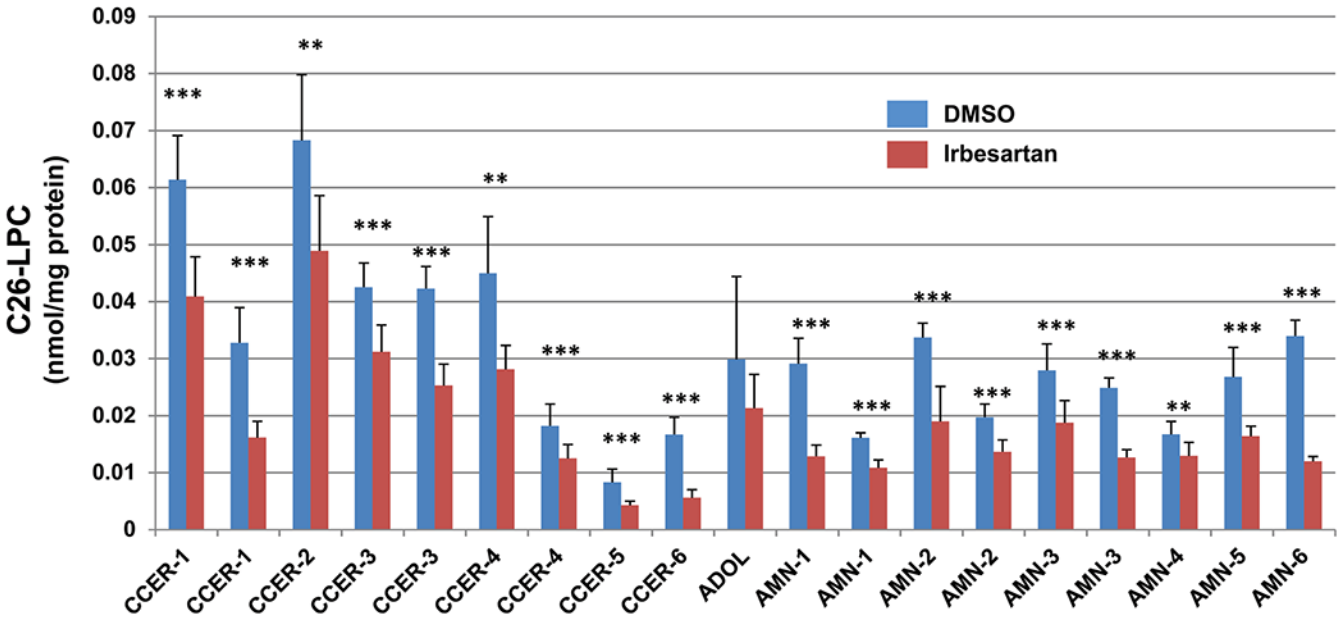

FIGURE 4.

Irbesartan consistently lowers C26-LPC levels in primary XALD fibroblasts.

Primary skin fibroblasts from CCER ($n = 6$), AMN ($n = 6$) and adolescent cerebral (ADOL; $n = 1$) XALD patients were treated with 10 μM irbesartan or vehicle (DMSO) for 10 days. Control fibroblasts were treated with vehicle alone. The C26-LPC content of 8 wells of a 96-well plate was quantitated and normalized to cellular protein as measured in a duplicate plate. Mean ± *SD* for each independent analysis is shown. Lowering of C26-LPC was statistically significant for all but the ADOL cell line (**$p < .01$; ***$p < .001$). AMN, adrenomyeloneuropathy; DMSO, dimethyl sulfoxide; XALD, X-linked adrenoleukodystrophy

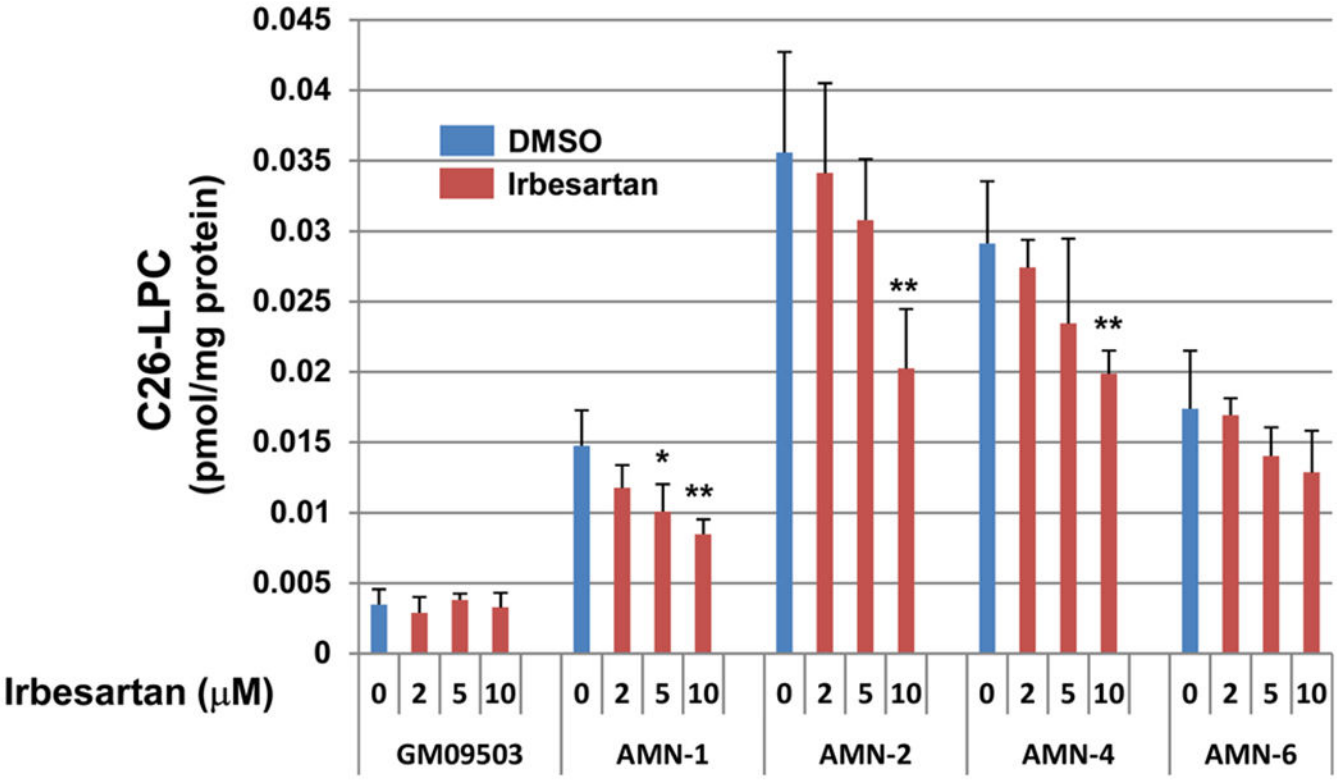

FIGURE 5.

Lowering of C26-LPC levels in primary XALD fibroblasts by irbesartan is dose-dependent. Primary fibroblasts from a normal control and four XALD patients were treated with 0–10 μM irbesartan for 10 days as described in Methods. The C26-LPC content of 4 wells of a 96-well plate was quantitated and normalized to cellular protein as measured in a duplicate plate. The final concentration of the vehicle, DMSO, was 0.1% for all wells. Mean ± *SD* (pmol C26-LPC/mg protein) is plotted. For AMN-1, AMN-2, and AMN-4, the C26-LPC level in drug-treated cells versus vehicle-treated cells was significantly lower at 10 μM irbesartan (**$p < .01$). The reduction in C26-LPC in AMN-1 cells was also significant at 5 μM irbesartan (*$p < .05$).

AMN, adrenomyeloneuropathy; DMSO, dimethyl sulfoxide; XALD, X-linked adrenoleukodystrophy

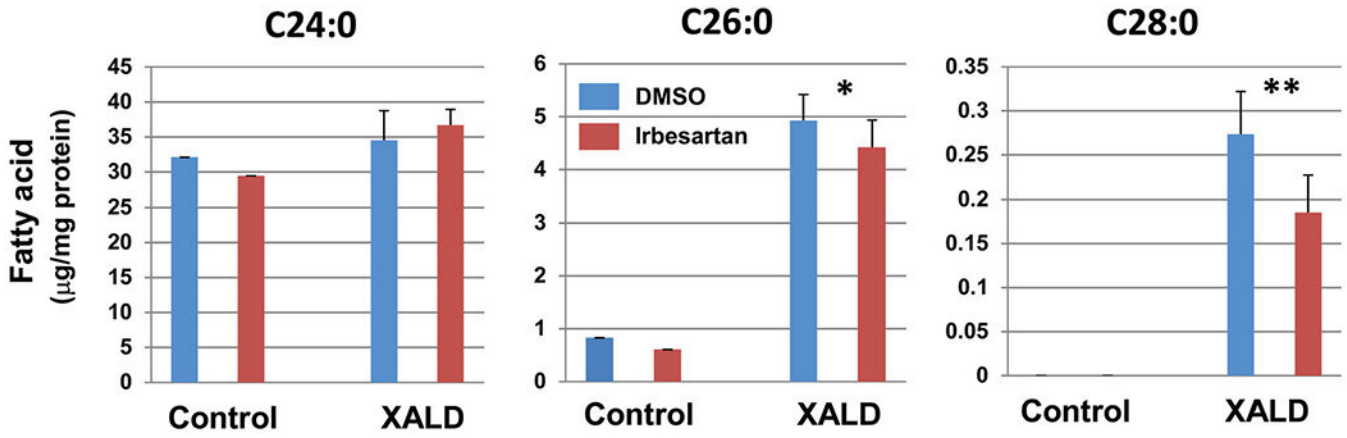

FIGURE 6.

Total VLCFA levels in primary fibroblasts treated with irbesartan. Duplicate 6 cm culture plates of one control, and four different XALD primary fibroblast, cell lines were treated with 10 μM irbesartan or vehicle (DMSO) alone for 10 days, after which total lipid fatty acids were quantitated by GC-MS as described in Methods. For XALD cells, mean ± *SD* is plotted. Irbesartan caused a statistically significant decrease in total C26:0 (*$p < .05$) and C28:0 (**$p < .01$), but not C24:0. Control cell C28:0 levels were below the level of detection. DMSO, dimethyl sulfoxide; VLCFA, very long-chain fatty acids; XALD, X-linked adrenoleukodystrophy

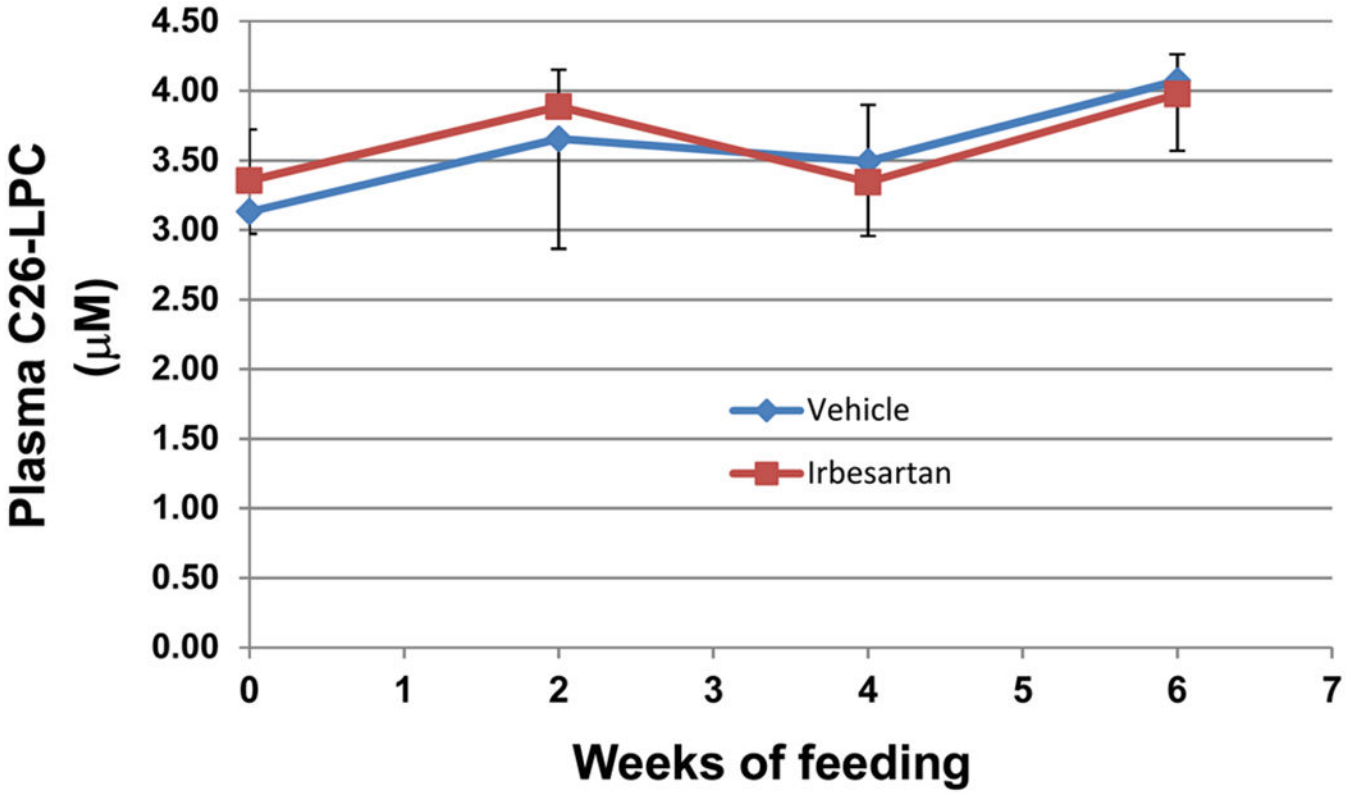

FIGURE 7.

Effect of oral irbesartan administration on plasma C26-LPC in XALD mice. Eight male XALD (hemizygous) mice were randomly placed into two groups of four. One group received chow containing adsorbed irbesartan calculated to deliver a dose of 10 mg/kg/day. The other group received chow containing adsorbed/evaporated vehicle (ethanol) only. Fresh food containing either irbesartan or no drug was provided twice weekly. At 0, 2, 4, and 6 weeks of treatment, plasma was obtained and C26-LPC was determined. Mean ± *SD* from each group of four mice is shown. XALD, X-linked adrenoleukodystrophy

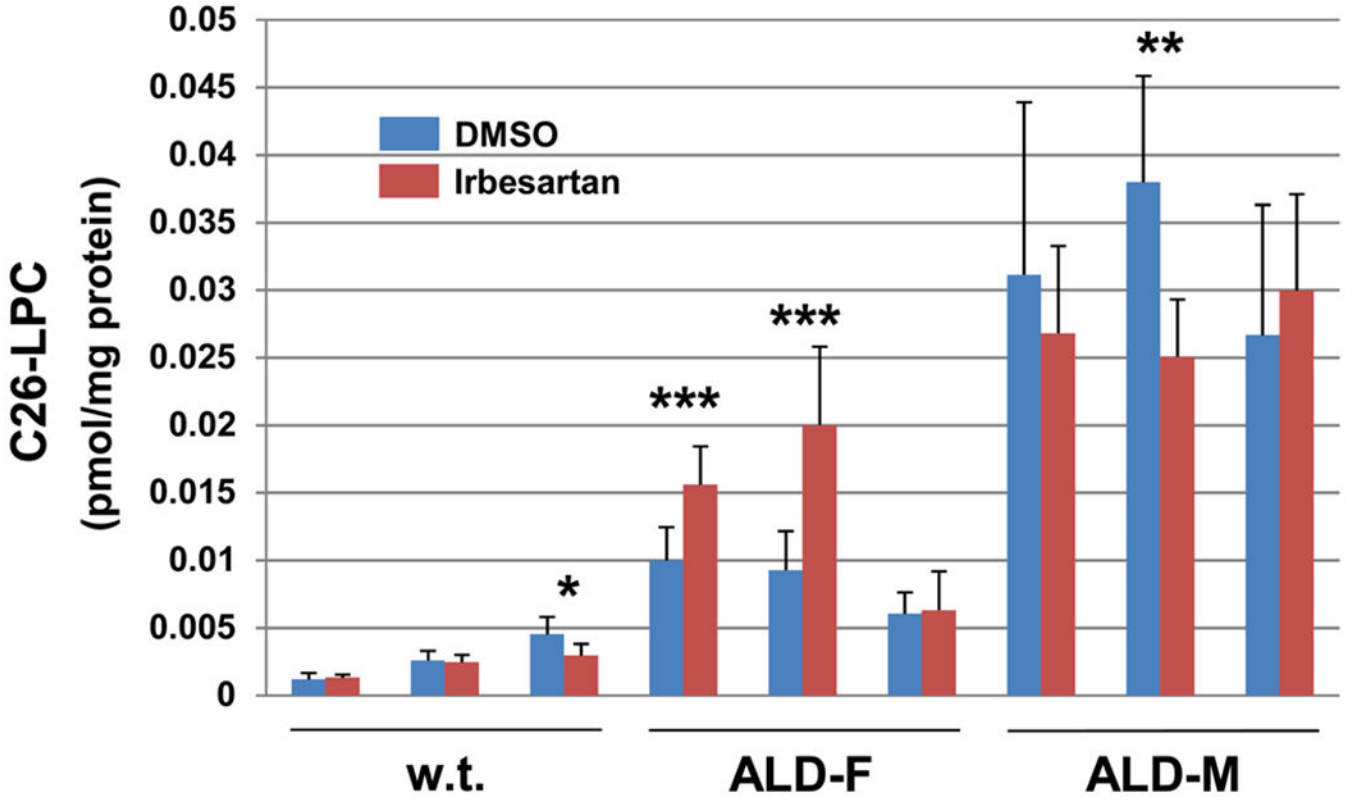

FIGURE 8.

C26-LPC levels in primary XALD mouse fibroblasts treated with irbesartan. Primary skin fibroblasts were prepared from male wild-type (w.t.) male mice, homozygous female (ALD-F) mice, and hemizygous male (ALD-M) mice. Fibroblasts grown in 96-well plates were treated with vehicle (DMSO) or 10 μM irbesartan for 10 days and C26-LPC was determined. Mean ± *SD* of octuplicate wells from each of three independent experiments is shown (*$p < .05$; **$p < .01$; ***$p < .001$). DMSO, dimethyl sulfoxide; XALD, X-linked adrenoleukodystrophy

TABLE 1

VLCFA levels in primary and immortalized normal control and XALD fibroblasts

Cells	Type	C26-LPC (pmol/mg protein)	Total C26:0 (μg/mg protein)	*n*
Normal	Primary	6.72 ± 1.92	0.062 ± 0.017	23
XALD	Primary	45.3 ± 16.8	0.40 ± 0.16	11
GM09503*E*	Immortalized	10.9 ± 2.5	n.d.	48
AMN-7*E*	Immortalized	43.1 ± 5.7	n.d.	48

Note: C26-LPC and total C26:0 were quantitated in cells grown to confluence in T-25 flasks (primary fibroblasts) or 96-well plates (immortalized fibroblasts) as described in Methods. Mean ± *SD* is shown.

Abbreviations: n.d., not determined; XALD, X-linked adrenoleukodystrophy.

TABLE 2

Change in C26-LPC level in primary XALD fibroblasts treated with drugs that lowered VLCFA levels in immortalized XALD fibroblasts

Cell line	% Decrease in C26-LPC/mg protein				Significance (Irbesartan)
	Dicoumarol	Papaverine	Thalidomide	Irbesartan	
CCER-1	10	−39	−9	42[a]	***, ***
CCER-2	−57	−2	30	28	**
CCER-3				33[a]	***, ***
CCER-4	−17	−18	−1	34[a]	**, ***
CCER-5				49	***
CCER-6				67	***
CCER-7	−3	−8	−4		
ADOL	10	1	5	29	**n.s.**
AMN-1				44[a]	***, ***
AMN-2				37[a]	***, ***
AMN-3				41[a]	***, ***
AMN-4				22	**
AMN-5				39	***
AMN-6				54[a]	***, ***
Mean	−11	−13	4	40	

Note: Candidate VLCFA-lowering drugs dicoumarol, papaverine, thalidomide, and irbesartan were tested at a concentration of 10 μM in multiple primary XALD cell lines. All cells were tested in octuplicate wells of a 96-well plate. In all cases, duplicate 96-well plates were prepared and treated identically. After 10 days of drug or vehicle (DMSO) treatment, one plate was used for C26-LPC analysis while the other was used to measure cellular protein. The C26-LPC values were normalized for cell protein before calculating the percent decrease caused by drug treatment. A negative value indicates an increase in C26-LPC. Decreases were not statistically significant for dicoumarol, papaverine, or thalidomide. Several cell lines were treated with irbesartan on two separate occasions, and the average percent decrease in C26-LPC is shown. The last column lists the statistical significance for each independent irbesartan study

** $p < .01$;

*** $p < .001$;

n.s., not significant.

Abbreviations: VLCFA, very long-chain fatty acids; XALD, X-linked adrenoleukodystrophy

[a]Average of two independent octuplicate analyses.